Reference: *Biol. Bull.* **174**: 153–162. (April, 1988)

Temperature Sensitivity of Molluscan and Arthropod Hemocyanins

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Abstract. The temperature sensitivity of hemocyanin-oxygen affinity and cooperativity was measured at 5, 15, 25, and 35°C in a variety of marine molluscs and arthropods from different thermal environments. These environments included a subtidal habitat in which the temperature is generally less than 15°C and the diurnal temperature variation is small, and an intertidal habitat in which the temperature varies more than 30°C. The temperature sensitivity of P_{50} showed considerable variation ($\Delta H = 0$ to $\Delta H = -67$ kJ/mol) depending on species and experimental temperatures. Sensitivity generally decreased as temperature increased. In several species temperature sensitivity was either absent or greatly reduced above 15°C. The horseshoe crab *Limulus polyphemus* showed a minimum temperature sensitivity between 15 and 25°C but higher sensitivity above and below this range. The hypothesis that a greater interaction between hemocyanin molecules and calcium ions at high temperatures offsets the temperature effect, resulting in a pigment less sensitive to temperature, was supported in an experiment where calcium ions were removed. Finally, delipidation of hemocyanin resulted in little or no change in oxygen affinity at all temperatures investigated.

Introduction

Long ago it was shown that O_2 affinity of the hemocyanins (Hcs), like almost all other O_2 carriers, decreases as temperature rises (Redfield, 1934). The range of temperature sensitivity is quite large, however, and exceptions to the general rule are known. While Miller and Van Holde (1981) retracted an earlier report of reversed temperature sensitivity of thalassinid Hc at low temperature, Morris *et al.* (1985) and Sanders and Childress (1985) both reported decreases in the O_2 affinity of other crustacean Hcs at low temperature. As is also true of most other O_2 carriers, temperature sensitivity of HcO_2 affinity varies within a species, often becoming smaller as temperature rises (Mauro and Mangum, 1982a, b; Bridges, 1986). In at least two species of terrestrial crustaceans, however, the temperature sensitivity of HcO_2 affinity is smallest in the temperature range in which the animals live (Morris and Bridges, 1985, 1986). Thus, the temperature sensitivity of HcO_2 affinity would appear to be variable and, at least occasionally, adaptive.

Oddly, few investigators have reported the effects of temperature on the cooperativity of HcO_2 binding. Mauro and Mangum (1982a, b) found the expected increase with temperature as molecular structure becomes less closed; the same trend appears to be present in the two curves shown by Angersbach and Decker (1978). However, no clear trend can be discerned in data shown by Jokumsen *et al.* (1981) and Bridges (1986).

In none of these investigations have temperatures above 25°C been examined, and yet many Hc-containing species do, in fact, experience such high temperatures. Moreover, all of the information summarized above pertains to crustacean Hcs. The few data available for chelicerate and molluscan Hcs (reviewed by Redfield, 1934; Mangum, 1980) suggest the same trends of an inverse relationship between temperature and its effects on HcO_2 affinity, and a direct relationship between temperature and its effects on cooperativity.

In an attempt to characterize the general thermal behavior of the hemocyanins, at least as a starting point, we have investigated the effects of temperatures from 5

Received 6 April 1987; accepted 25 January 1988.

to 35°C on HcO_2 affinity and cooperativity, using both arthropod and molluscan Hcs and choosing species representing two quite distinct thermal environments. One group, designated cold water species, consists of subtidal crabs and an abalone collected from coastal waters of southern California and Puget Sound where seasonal temperature changes are small. The other group, designated the eurythermal species, consists of intertidal (at least transiently) and semi-terrestrial arthropods and molluscs, which originated from a variety of localities where the temperature is generally higher and quite variable.

We have also tested further the hypothesis that a lipid moiety of hemocyanin is related to the temperature dependence of HcO_2 binding (Mangum *et al.*, 1987), since it has been suggested previously that the lipid moiety of Hc influences O_2 binding in a way that might explain the seasonal change reported by several investigators (Zatta, 1981; Mauro and Mangum, 1982a). Finally, since ionic activity also increases with temperature, we have examined the role of the allosteric modulator Ca^{+2} in the temperature dependence of HcO_2 binding.

Materials and Methods

Experimental animals

The cold water species, represented by the crabs *Cancer anthonyi* (Rathbun), *C. gracilis* (Dana), and *Lopholithodes foraminatus* (Stimpson), and the pink abalone *Haliotis corrugata* (Gray), were held in running seawater at approximately 15°C. Hemolymph from another cold water crab, *C magister* (Dana), was kindly furnished by D. D. Jorgensen. Individuals of the most terrestrial (and therefore, eurythermal) crustacean studied, *Eurytium albidigitum* (Rathbun), were obtained from the northern Gulf of California at Laguna Percebu, 16 km south of San Felipe, Baja California, Mexico, and transported to San Diego, where they were held at 23°C. This species experiences temperatures ranging from about 15 to 36°C (Burnett and McMahon, 1987). Hemolymph was sampled from the intertidal chiton *Stenoplax conspicua* (Carpenter) *in situ* at Bird Rock, San Diego, where the air temperature was about 23°C and the water temperature 15°C. This eurythermal species experiences temperatures ranging from about 11 to 25°C. The chelicerate *Limulus polyphemus* (Linnaeus) was collected from the seaside coast of Virginia and held in recirculating seawater at 18–20°C. This species, which becomes intertidal only during its spring migrations into the estuary, experiences air temperatures ranging up to 30°C and water temperatures below 5°C.

O_2 equilibria

Hemolymph was sampled from the animals held under the conditions described above and oxygen equilibrium curves were determined at 5, 15, 25, and 35°C (except where noted) using techniques described below.

O_2 equilibria of *L. polyphemus* Hc were obtained by the cell respiration method (Mangum and Lykkeboe, 1979). All other data were collected tonometrically (Burnett, 1979; Burnett and Infantino, 1984). Temperature was controlled in all cases using thermostated water baths ± 0.1°C. The hemolymph samples from arthropods were allowed to clot and the clot disrupted using a glass homogenizer. All samples were centrifuged and 0.1 ml of the supernatant was added to 4.5 ml buffered saline. In two cases Hc concentration was low and required the addition of larger volumes of the supernatant (0.5 ml for *H. corrugata* Hc and 1 ml for *C. magister* Hc). The preparations were equilibrated to mixtures of N_2 (99.99% pure and <0.05 ppm O_2) and either air scrubbed of CO_2 and water, or O_2 (estimated 99.7% pure). Percent HcO_2 was estimated at 345 nm (Bausch & Lomb Spectronic 21 colorimeter).

The physiological salines used for *C. anthonyi, C. gracilis, C. magister,* and *L. foraminatus* Hcs were the same as that used earlier for *C. anthonyi* Hc (Burnett and Infantino, 1984). The saline used for *E. albidigitum* Hc was the same as that used for *Uca princeps* Hc (Burnett and Infantino, 1984), and the saline for *S. conspicua* Hc was that used for *Cryptochiton stelleri* Hc (Mangum and Burnett, 1986). The salines were buffered with either 0.05 mol/l HEPES, using HCl or NaOH to adjust pH, or 0.05 mol/l Tris maleate.

The data were described by regression lines (pH *versus* log P_{50}) and, if the slopes were homogeneous, differences in the Y intercepts assessed by analysis of covariance. In addition, the slopes of the regression lines describing log P_{50} as a function of pH were tested for differences from zero using a student's *t*-test. Temperature sensitivity of oxygen affinity was analyzed using van't Hoff plots where log P_{50} is plotted against 1/T (in degrees Kelvin) and the resulting slope is proportional to the heat of oxygenation, ΔH, *i.e.* slope = ΔH/gas constant. The data used for these plots were obtained from regression analysis of pH *versus* log P_{50} at different temperatures. This method of analysis allowed us to determine the effects of temperature on oxygen affinity at constant pH. In some cases positive values for ΔH resulted, which we attribute to data scatter especially in the cases of *C. gracilis* and *L. foraminatus.*

Sera were delipidated as described by Mangum *et al.* (1987). 0.02 g Triton X 100/g Hc [estimated by measuring the absorbance of hemolymph diluted with 10 mmol/l EDTA at pH 8.9 to eliminate light scattering and

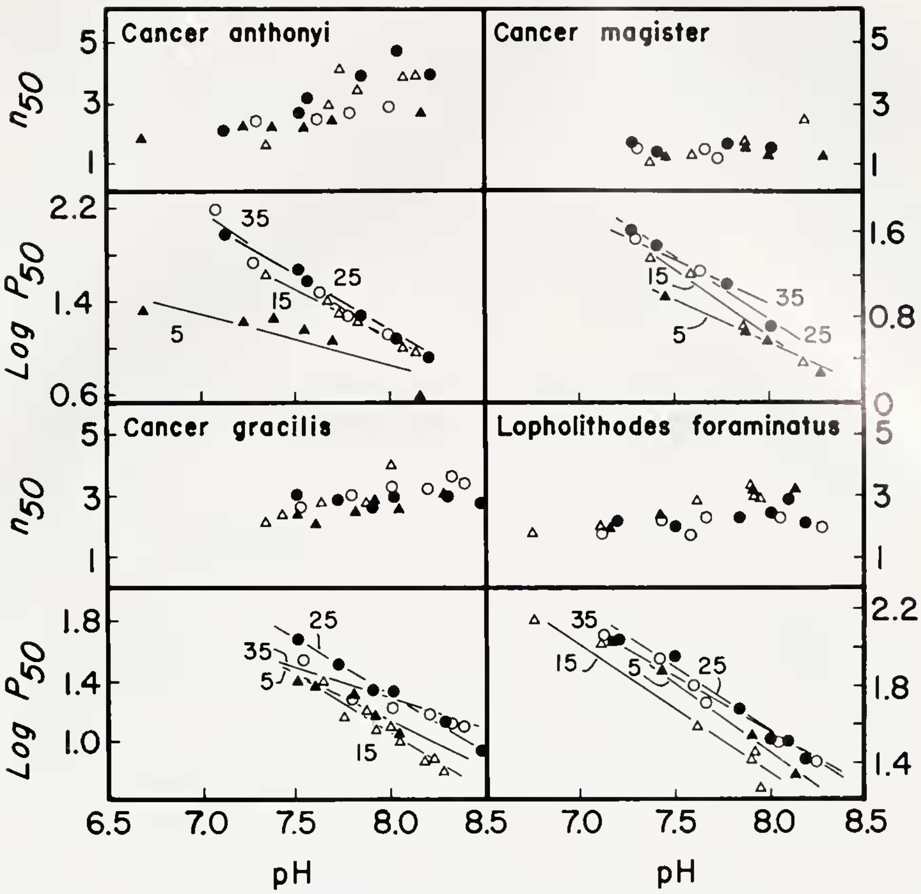

Figure 1. The effect of temperature on hemocyanin oxygen affinity (log P_{50}) and cooperativity (n_{50}) as a function of pH in four "cold" water crabs. *Cancer anthonyi, Cancer magister, Cancer gracilis,* and *Lopholithodes foraminatus.* P_{50} and n_{50} were determined at 5°C (▲), 15°C (△), 25°C (●), and 35°C (○).

using extinction coefficients reported by Nickerson and Van Holde (1971)] was combined with serum and stirred at room temperature for 1 h. The lipid-detergent complex was removed by adding 1 g Bio-Beads (SM-2 20-50 mesh. BioRad Co.)/g Hc and stirring for another h. The Bio-Beads were then removed by filtration through cotton.

Calcium was removed from samples of *C. anthonyi* hemolymph by dialysis overnight against two changes of 500 mmol/l NaCl and 1 mmol/l EGTA (ethyleneglycol-bis-N,N-tetra-acetic acid) in a ratio of 1 volume of sample:500 volumes of dialysis medium.

Results

In five of the eight species studied (the exceptions being *S. conspicua, L. foraminatus,* and *L. polyphemus*) temperature sensitivity of HcO_2 affinity is generally lowest at the highest temperatures (Figs. 1, 2, 3). In the van't Hoff plots (Fig. 3) this is represented by slopes which approach zero. In all species the Bohr coefficients (Δ log $P_{50}/\Delta pH$) differ significantly from zero ($P < .01$) throughout the temperature range.

In the five cold water species, temperature sensitivity of P_{50} is absent or, in *H. corrugata*, lowest between 25 and 35°C (Figs. 1, 2; Table I). This is most easily seen in the van't Hoff plots (Fig. 3). HcO_2 affinity in *C. gracilis* and *C. magister* also does not change significantly from 5 to 15°C at the physiological pH (7.8) (Table I). Surprisingly, a large positive value for ΔH was found in *L. foraminatus* between 5 and 15°C. However, if ΔH is calculated for the interval between 5 and 25°C, small negative values between −6.7 and −8.3 kJ/mol over the pH range result.

In contrast, HcO_2 affinity in the [illegible] eurythermal species changed significantly with temperature throughout the range examined with the exception of *E. albidigitum* at low pH (Fig. 2; Table I). The Hc of *E. albidigitum* also showed the trend of decreasing temperature sensitivity at

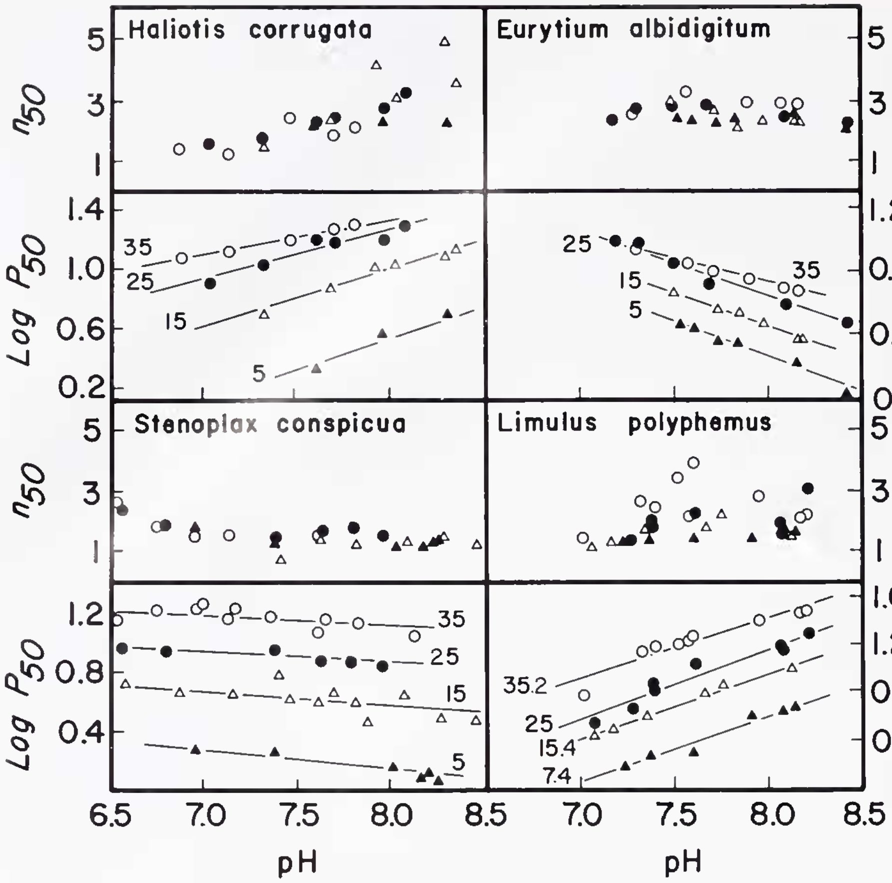

Figure 2. The effect of temperature on hemocyanin oxygen affinity (log P_{50}) and cooperativity (n_{50}) as a function of pH in the abalone *Haliotis corrugata*, the chiton *Stenoplax conspicua*, the xanthid crab *Eurytium albidigitum*, and the horseshoe crab *Limulus polyphemus*. P_{50} and n_{50} were determined at 5°C (▲), 15°C (△), 25°C (●), and 35°C (○), except as noted for *L. polyphemus*.

higher temperatures. In *L. polyphemus* minimal sensitivity was found in the 15 to 25°C range. Later it was found that this phenomenon is due to complete insensitivity between 20 and 25°C and that the ΔH value for 15–20°C is unexceptional (C. P. Mangum and J. Ricci, in prep.).

The values for cooperativity of the arthropod Hcs show some tendency to increase in the middle and upper ends of the pH range examined (Figs. 1, 2). In *C. anthonyi* and *H. corrugata* the pH dependence of cooperativity appears to increase with temperature; in the other species there is no clear trend. In general, however, cooperativity is influenced very little by either pH or temperature. Like other polyplacophoran Hcs (*e.g.*, Mangum and Burnett, 1986), *S. conspicua* Hc exhibits very little cooperativity, a feature that does not change with temperature (Fig. 2). Above 5°C, cooperativity of *H. corrugata* Hc decreases with decreasing pH (Fig. 2), a finding that agrees with Ainslie's (1980) report of a decrease in n_{50} with increasing P_{CO_2} of three other *Haliotis* Hcs. Otherwise temperature has no clear effect.

Dialyzing *C. anthonyi* Hc against 500 mmol/l NaCl and 1 mmol/l EGTA caused large decreases in O_2 affinity (*cf.* Figs. 1, 4). The present results indicate that Ca^{+2} has an effect on temperature sensitivity. The temperature sensitivity of the Hc dialyzed against a calcium-free saline and EGTA was slightly less than the controls between 5 and 25°C but much greater than the controls between 25 and 35°C (Fig. 5). Between 5 and 25°C the differences between ΔH were greatest at low pH and opposite to that predicted by our hypothesis (see Discussion).

At the two temperatures investigated, delipidation of *C. anthonyi* and *S. conspicua* Hcs caused no significant changes in O_2 affinity or its temperature sensitivity (Fig. 6; Table II). This result agrees with an earlier finding for *Callinectes sapidus* Hc (Mangum *et al.*, 1987). Delipidation of *E. albidigitum* Hc appears to have induced a small but significant decrease in O_2 affinity. We view this result with caution, however, in part because it is opposite to the change reported by Zatta (1981) and in part because at 35°C it occurs only at high pH.

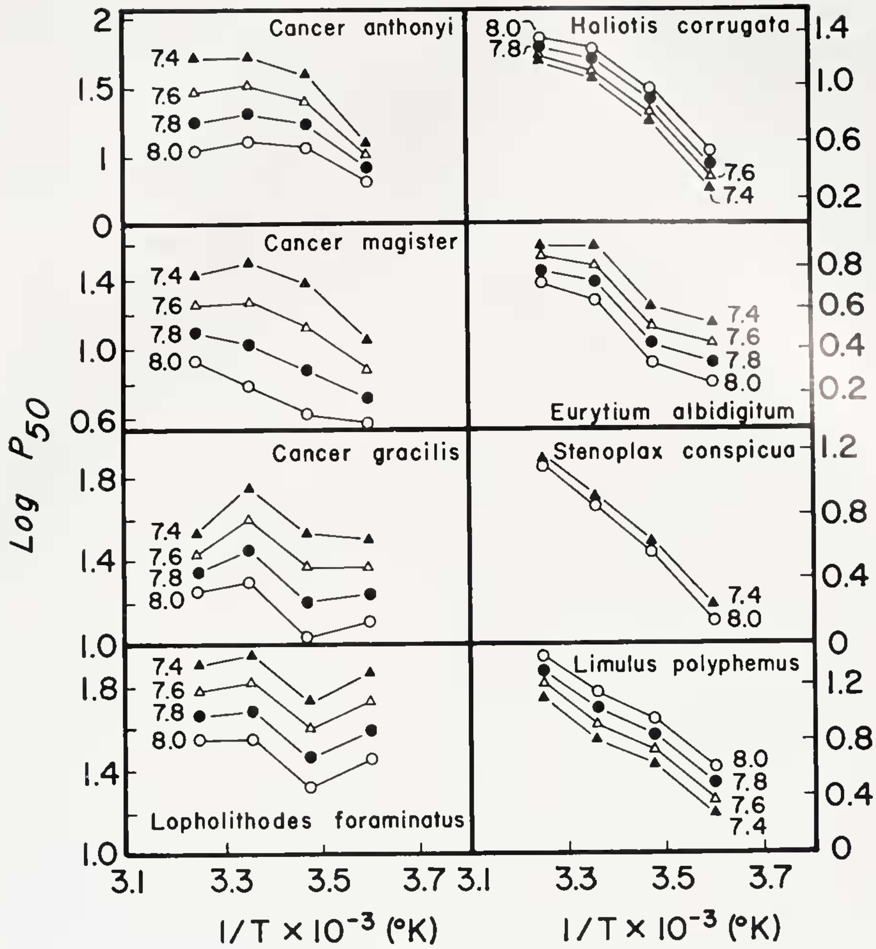

Figure 3. The effect of temperature on oxygen affinity at different pH expressed as van't Hoff plots: pH 7.4 (▲), pH 7.6 (△), pH 7.8 (●), pH 8.0 (○).

Discussion

In an evolutionary sense the O_2 binding properties of the crustacean Hcs have been considered rather conservative relative to those of some of the other O_2 carriers (Mangum, 1980). However, HcO_2 affinities are adaptable, both genetically and non-genetically. Of particular relevance here, higher O_2 affinities are found in species inhabiting warmer waters and lower O_2 affinities in species inhabiting colder waters (Redmond, 1968; Mangum, 1982; Mauro and Mangum, 1982b). The difference is adaptive because it offsets, in part, the intrinsic influence of temperature, *viz.* a decrease in HcO_2 affinity as temperature rises. The genetic adaptation enhances deoxygenation at the tissues in cold water species and oxygenation at the gill in warm water species. The latter may be especially important in species that encounter air (and must enhance the waterproofing of the cuticle, thus also increasing diffusion resistance of the gas exchanger) and in species that encounter hypoxic water. The adaptation is not perfect, however, and the available evidence indicates that the HcO_2 transport system does not play as large a role at low temperatures as it does at high temperatures (Mangum, 1980; Mauro and Mangum 1982b). The present results confirm the finding that the smaller role of the system at low temperature is due to a widespread increase in temperature dependence of O_2 binding.

A question arises as to why the various species have such different thermal sensitivities. ΔH values range from 0 to −70 kJ/mol in our sample and a [illegible] greater range is found in a larger sample (see Introduction). The answer may lie in the relationship between the relative magnitudes of temperature and pH dependence of the Hc, which appear to be inversely related. When the Bohr shift is normal and large, temperature dependence is small (*e.g.*, the intertidal species in the present sample) and vice versa (the cold water species in the present sample).

Table I

The effect of temperature on HcO_2 oxygen affinity

		log P_{50} vs. pH			
			Analysis of covariance		
		Bohr coeff.	Slope	Y-intcpt	ΔH (kJ/mol)
Cancer anthonyi	5	−0.49	NS	**	−49.2
	15	−0.88	*	NA	−13.3
	25	−1.02	NS	NS	10.0
	35	−1.11			
Cancer magister	5	−0.85	NS	NS	−25.1
	15	−1.25	NS	*	−24.7
	25	−1.18	NS	NS	−11.4
	35	−0.83			
Cancer gracilis	5	−0.65	NS	NS	5.7
	15	−0.82	NS	**	−41.4
	25	−0.75	**	NA	18.1
	35	−0.46			
Lopholithodes foraminatus	5	−0.70	NS	*	19.3
	15	−0.69	NS	NS	−36.9
	25	−0.67	NS	NS	4.0
	35	−0.60			
Haliotis corrugata	5	0.25	NS	**	−72.4
	15	0.35	NS	**	−46.2
	25	0.39	NS	**	−15.9
	35	0.44			
Eurytium albidigitum	5	−0.48	NS	**	−29.7
	15	−0.46	NS	**	−31.2
	25	−0.43	*	NA	−10.3
	35	−0.32			
Stenoplax conspicua	5	−0.15	NS	**	−64.4
	15	−0.12	NS	**	−47.3
	25	−0.07	NS	**	−43.2
	35	−0.09			
Limulus polyphemus	7.4	0.56	NS	**	−67.4
	15.4	0.53	NS	**	−33.0
	25	0.67	NS	**	−47.7
	35.2	0.05			

The analysis of covariance tests for differences in the slopes of regression lines fit to the data describing P_{50} as a function of pH for each Hc. If the slopes did not change with temperature, the analysis of covariance was used to test for differences in the Y-intercepts of the lines. ΔH was calculated from the value predicted by the regression analysis at pH 7.8. All slopes were different from zero according to a student's *t*-test. * = $.01 < P < .05$; ** = $P < .01$; NS = no significant difference at 0.05 level; NA = not applicable since slopes are significantly different.

When pH dependence is large, low temperature sensitivity minimizes the indirect effect of temperature due to the thermal sensitivity of hemolymph pH. The net effect of these interactions is to minimize the changes in temperature and oxygen affinity within a species. This is seen in Figure 7 where P_{50} is plotted as a function of temperature and pH is allowed to vary with temperature. While the absolute P_{50} may be dissimilar between species, the slope of the relationship between temperature and oxygen affinity within a species is relatively constant. Thus, oxygen affinity changes due to temperature are similar between species regardless of habitat. We emphasize that the relationship between hemolymph pH and temperature has not been measured in these six species but instead was predicted from the quantity $\Delta pH/\Delta °C = -0.016$ (Truchot, 1983); a number of exceptions to the rule are known (*e.g.*, Polites and Mangum, 1980). Figure 7 is intended only to illustrate the stabilizing potential of the interaction between pH and temperature sensitivities. This pattern appears to characterize at least some other Hcs with normal Bohr shifts (other studies). For example, the Hcs of the crabs *Uca princeps* and *Callinectes bellicosus* have moderate to large Bohr shifts (−0.71 and −1.32, respectively) and are relatively insen-

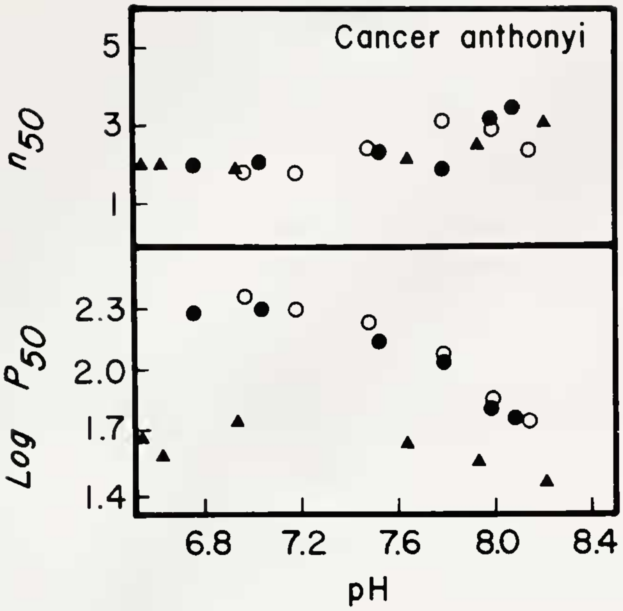

Figure 4. Oxygen affinity (log P_{50}) and cooperativity (n_{50}) of *Cancer anthonyi* hemocyanin in the absence of calcium ions as a function of pH and temperature; 5°C (▲), 25°C (●), and 35°C (○). Calcium ions were removed from the hemocyanin by dialyzing against 500 mmol/l NaCl and 10 mmol/l EGTA.

sitive to temperature (Burnett and Infantino, 1984). The freshwater crab *Holthuisana transversa* has a very small Bohr shift (<−0.2) and a pronounced temperature dependence (Morris *et al.*, 1988). However, there are also a number of Hcs with large normal Bohr shifts and a conventionally large temperature dependence as well (Jokumsen *et al.*, 1981; Mauro and Mangum, 1982a, b; Bridges *et al.*, 1983; Morris and Bridges, 1985, 1986); there must be additional selection pressures for Bohr shifts that may override the adaptive potential described here.

An indirect mechanism may be responsible for the decrease in temperature dependence at high temperatures which we have noted in some species. Andersson *et al.* (1982) showed that the number of available binding sites for the allosteric modulator Ca^{+2}, which raises O_2 affinity of Hcs with normal Bohr shifts, increases with temperature. One might also expect an increase in Ca^{+2} affinity of Hc at high temperature due to enhanced ionic activity and possibly other factors associated with changes in protein structure. We reasoned, therefore, that a greater interaction between calcium ions and Hc at higher temperature (*i.e.*, greater Ca^{+2} activity and more Ca^{+2}-Hc binding sites) would tend to increase oxygen affinity [provided that these Hcs respond to changes in free calcium ions as do those of other portunid crabs (Truchot, 1975; Mason *et al.*, 1983)] at a time when temperature acts to decrease it. The net result would be a depression of the temperature effect at higher temperature. This was indeed observed in our experiments where we removed calcium ions. We should mention that very small amounts of Mg^{+2}, which generally has a stronger effect than Ca^{+2} (Mason *et al.*, 1983), may have been present in our preparations since we chose not to use EDTA. It seems unlikely, however, that trace amounts would substitute exactly for the Ca^{+2} removed, and it seems more likely that the typical pattern of temperature dependence is a direct and intrinsic feature of calcium ion activity and/or protein structure. Furthermore, the presence of small amounts of Mg^{++} probably served to prevent the dissociation of the dodecamers into subunits (Ellerton *et al.*, 1970). Significant light scattering due to the presence of large subunits was observed in our dialyzed, deoxygenated samples. The light scattering was quantitatively similar (accounting for 5 to 19% of the absorbance in oxygenated samples) to control samples.

The response of *L. polyphemus* Hc to high temperature is also consistent with the above hypothesis. Greater calcium ion activity has an opposite effect on oxygen affinity causing an increase in P_{50} (Diefenbach and Mangum, 1983). Thus, at high temperature an increase in Hc calcium binding along with higher Ca^{+2} activity results in a large temperature-induced decrease in O_2 affinity. However, the responses of Hc of the hermit crab *Coenobita clypeatus* to temperature and calcium do not support the hypothesis. *C. clypeatus* has a small normal Bohr shift and temperature sensitivities which are smaller between 25 and 30°C but greater between 30 and 35°C (Morris and Bridges, 1986). While this pattern of temperature responses is similar to that found in *L. polyphemus*, changes in calcium ion binding to Hc and increases in calcium activity with temperature cannot be used to ex-

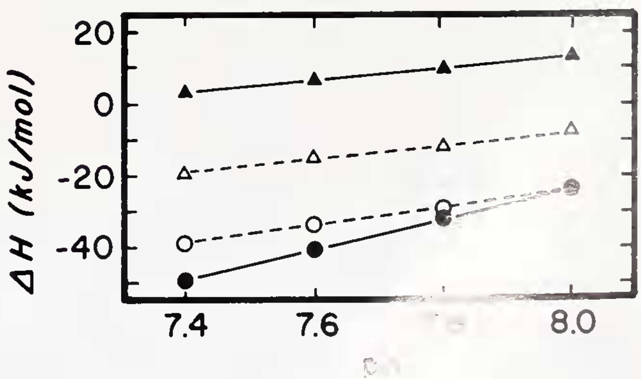

Figure 5. The heat of oxygenatio[illegible] *ancer anthonyi* hemocyanin between 5 and 25°C (circles [illegible]) and 35°C (triangles) as a function of pH. Control values are represented by closed symbols while open symbols represent samples dialyzed against 500 mmol/l NaCl and 10 mmol/l EGTA.

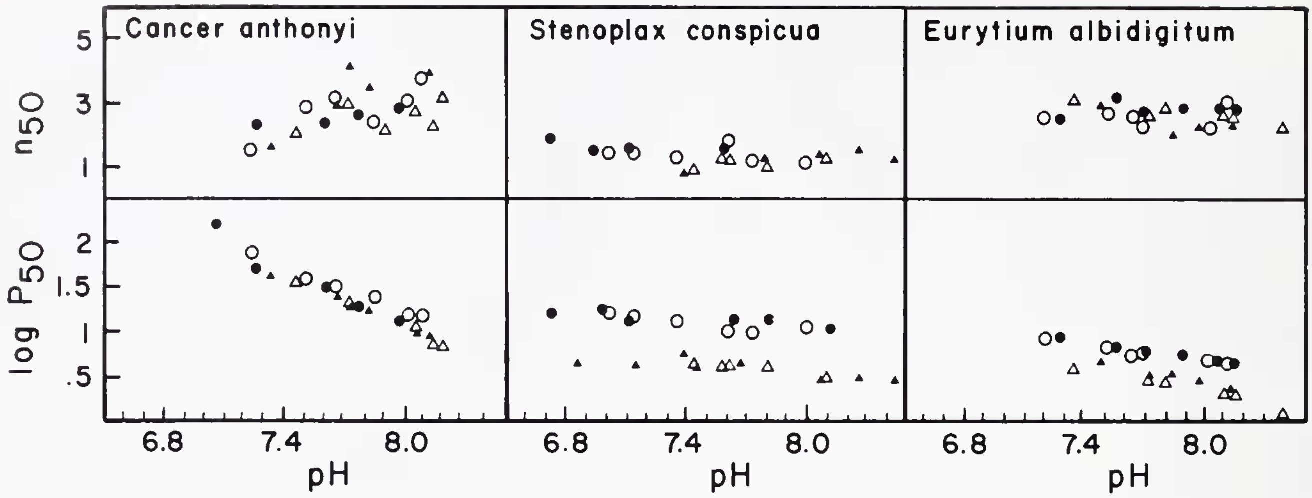

Figure 6. The effect of delipidation on *Cancer anthonyi*, *Stenoplax conspicua*, and *Eurytium albidigitum* hemocyanin oxygen affinity (log P_{50}) and cooperativity (n_{50}) as a function of pH and temperature; 15°C (triangles) and 35°C (circles). Values for delipidated hemocyanin are represented with open symbols; values for untreated hemocyanin are represented with closed symbols.

plain the increase in sensitivity at high temperature, since the oxygen affinity of *C. clypeatus* Hc is insensitive to changes in calcium ion concentration.

This hypothesis should be tested in future studies by measuring the temperature responses of oxygen affinity in Hcs from a variety of species that demonstrate a calcium sensitivity and from those that demonstrate no calcium sensitivity. If the presence of 3 to 17 low affinity calcium binding sites per subunit (Andersson *et al.*, 1982) is prevalent among the hemocyanins, it would in-

Table II

The effect of delipidation on the temperature dependence of HcO_2 binding

		n_{50} vs. pH					log P_{50} vs. pH		
				Analysis of covariance				Analysis of covariance	
	Temp.	Slope	Diff. from 0	Slope	Y-intercept	Bohr coeff.	Diff. from 0	Slope	Y-intercept
Cancer anthonyi									
control	15	2.74	*	NS	*	−0.88	**	NS	NS
delipidated	15	0.67	NS			−0.98	**		
control	35	0.63	NS	NS	NS	−1.11	**	NS	NS
delipidated	35	1.88	*			−0.80	**		
Eurytium albidigitum									
control	15	−0.86	NS	NS	*	−0.46	**	NS	**
delipidated	15	−0.70	*			−0.45	**		
control	35	0.15	NS	NS	NS	−0.32	**	NS	**
delipidated	35	0.16	NS			−0.29	**		
Stenoplax conspicua									
control	15	0.29	NS	NS	NS	−0.12	**	NS	NS
delipidated	15	0.17	NS			−0.18	**		
control	35	−0.88	NS	NS	NS	−0.09	**	NS	NS
delipidated	35	−0.20	NS			−0.20	**		

The slopes of regression lines fit to the data describing log P_{50} as a function of pH did not change with temperature or delipidation. The analysis of covariance was used to test for differences in Y-intercept values. * = $.01 < P < .05$; ** = $P < .01$; NS = no significant difference at 0.05 level.

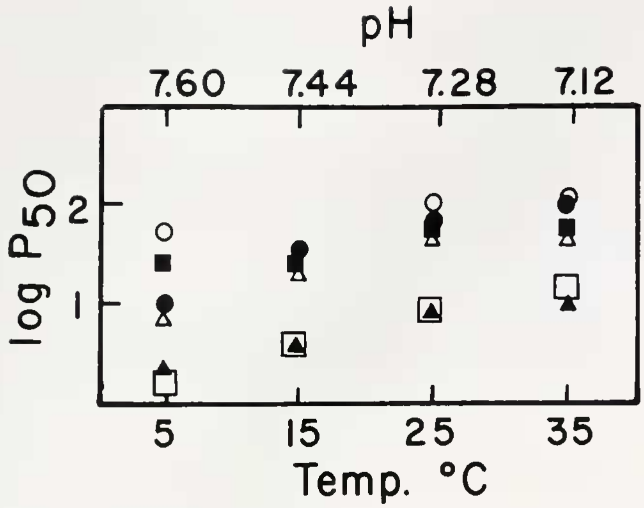

Figure 7. The relationship between temperature, where pH is assumed to vary according to ΔpH/Δ°C (Truchot, 1983), and the Bohr shift are plotted for *Stenoplax conspicua* (□), *Eurytium albidigitum* (△), *Cancer gracilis* (■), *Cancer magister* (▲), *Cancer anthonyi* (●), and *Lopholithodes foraminatus* (○).

dicate a new and physiologically important role for calcium in temperature responses.

Finally, in these species the lipid moiety of Hc apparently serves no purpose in stabilizing O_2 affinity over the thermal range investigated. These results are similar to those reported by Mangum *et al.* (1987), who showed that removal of serum lipids has no effect on oxygen binding in the crab *Callinectes sapidus.*

In summary, the effects of temperature on O_2 affinity of both arthropod and molluscan Hcs are highly variable. However, the most common pattern is greater sensitivity at low temperature and less sensitivity at high temperature. Furthermore, among the eight Hcs examined here, there was no sign of the reversed thermal sensitivity at low temperatures reported by Morris *et al.* (1985) and Sanders and Childress (1985), suggesting that it is not a particularly widespread adaptive mechanism.

Acknowledgments

L. Burnett was supported by a grant from Research Corporation. C. P. Mangum was supported by NSF DCB 84-14856 (Regulatory Biology).

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